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Cinnamophilin as a novel antiperoxidative cytoprotectant and free radical scavenger

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Abstract

The antioxidant properties of cinnamophilin were evaluated by studying its ability to react with relevant reactive oxygen species, and its protective effect on cultured cells and biomacromolecules under oxidative stress. Cinnamophilin concentration-dependently suppressed non-enzymatic iron-induced lipid peroxidation in rat brain homogenates with an IC_{50} value of 8.0 ± 0.7 μM and iron ion/ADP/ascorbate-initiated rat liver mitochondrial lipid peroxidation with an IC_{50} value of 17.7 ± 0.2 μM. It also exerted an inhibitory activity on NADPH-dependent microsomal lipid peroxidation with an IC_{50} value of 3.4 ± 0.1 μM without affecting microsomal electron transport of NADPH-cytochrome *P*-450 reductase. Both 1,1-diphenyl-2-picrylhydrazyl and 2,2′-azo-bis(2-amidinopropane) dihydrochloride-derived peroxyl radical tests demonstrated that cinnamophilin possessed marked free radical scavenging capacity. Cinnamophilin significantly protected cultured rat aortic smooth muscle cells (A7r5) against alloxan/iron ion/H_2O_2-induced damage resulting in cytoplasmic membranous disturbance and mitochondrial potential decay. By the way, cinnamophilin inhibited copper-catalyzed oxidation of human low-density lipoprotein, as measured by fluorescence intensity and thiobarbituric acid-reactive substance formation in a concentration-dependent manner. On the other hand, it was reactive toward superoxide anions generated by the xanthine/xanthine oxidase system and the aortic segment from aged spontaneously hypertensive rat. Furthermore, cinnamophilin exerted a divergent effect on the respiratory burst of human neutrophil by different stimulators. Our results show that cinnamophilin acts as a novel antioxidant and cytoprotectant against oxidative damage.

Keywords: Antioxidant; Lipid peroxidation; Peroxyl radical; Cytoprotectant; Superoxide anion; Cinnamophilin

1. Introduction

Accumulating evidence strongly suggests that free radicals and other oxygen-reactive species are the important causative factors of degenerative diseases, including aging [1], brain dysfunction [2], some hepatopathies [3] and cardiovascular disorders [4]. Oxidative stress occurs following either abnormal overproduction of reactive oxygen species or impairment of endogenous antioxidant defense systems. Under oxidant insult, various cellular/extracellular bioactive mediators and biomacromolecules were inactivated and deteriorated [5]. Consequently, the free radical itself directly or its oxidative by-products lead to results including disruption of cellular structures [5], alteration of activity of bioactive enzymes [6], expression of proinflammatory genes [7], alteration of cellular proliferation or death [8] and even dysfunction of endothelium-dependent vasodilation [9].

It has been suggested that the majority of biomacromolecules with highly polyunsaturated fatty acids, including the biomembranes and low-density lipoprotein (LDL), are susceptible to attack by reactive oxygen species and easily induced lipid peroxidation [10]. Lipid peroxidation not only causes disturbance in the structure of the membrane but also gives rise to reactive peroxidative products which are also capable of modification of proteins or DNA, thereby leading to short- or long-term cellular dysfunction [11]. Accumulating evidence suggests the involvement of

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oxidatively modified LDL in atherogenesis, although the formation of oxidized LDL in vivo remains unclear [12]. However, it is known that oxidized LDL are taken up by the macrophages and eventually converted to the lipid-laden foam cells. Oxidized LDL could induce a chronic inflammatory reaction, not only itself as a chemoattractant, but also as a cytotoxic substance [13]. According to in vitro and in vivo studies, several classic antioxidants are suggested to protect LDL against lipid peroxidation, and therefore to prevent the occurrence of atherogenetic events [14].

Recently, an important issue relevant to enhanced superoxide anion production is the novel pathogenetic role in adult respiratory distress syndrome [15], diabetic vascular disease [16], angiotensin-induced or generic hypertension [17] and ischemia-reperfusion injury [18]. It is well known that superoxide anion is augmentedly formed during mitochondrial dysfunction [19], catalysis by xanthine oxidase [20] and NADPH oxidase from activated phagocytic [21] or vascular cells [22], and even tetrahydrobiopterin-deficient nitric oxide synthase [23]. Especially, since superoxide anions essentially react with bioactive nitric oxide, increased vascular superoxide production has been proposed to account for the blunted vasodilator response in some cardiovascular disorders [23]. And, consequentially, its derived products, including peroxynitrite and hydrogen peroxide, as permeant and potent oxidants could also mediate in vasculopathies as vascular cell injury [24].

Cinnamophilin (Fig. 1), a natural compound isolated from *Cinnamomum philippinense* [25], possesses both thromboxane A_2 synthase inhibitory and thromboxane A_2 receptor antagonizing properties [26]. Interestingly, this new lignan also could abrogate autooxidation in a preliminary study [25]. Recently, it has been reported that cinnamophilin is effective in the reduction of reperfusion-induced arrhythmia [27]. In the present study, we have investigated the antioxidant activity of cinnamophilin as revealed by its protective effect against various free radicals or lipid peroxidation in some in vitro models. Especially, we used the flow cytometric method to assess its cytoprotective and mitochondrial stabilizing actions under oxidative stress. Additionally, the antiperoxidative capability of cinnamophilin to its anti-LDL oxidative action is investigated. Furthermore, our experiments were also aimed at whether cinnamophilin could ameliorate superoxide anion production in the vessels from aged spontaneously hypertensive rat (SHR) or stimulated human neutrophils.

2. Materials and methods

2.1. Chemicals and reagents

Cinnamophilin was isolated from the root of *C. philippinense* as previously described [25] and dissolved in dimethyl sulfoxide (DMSO). In each experimental method, DMSO was employed at a constant final concentration. In the present study, the purity of cinnamophilin was estimated to be $>99\%$ on the basis of ^{1}H-NMR (400–200 MHz), IR and MS (70 eV). Cytochrome *c* (type III, from bovine liver), probucol, α-tocopherol, butylated hydroxytoluene (BHT), desferrioxamine mesylate, 2-thiobarbituric acid, tetramethoxypropane, diphenyl-*p*-picrylhydrazyl, B-phycoerythrin, xanthine, xanthine oxidase (grade IV, from buttermilk), bovine serum albumin (BSA), ascorbic acid, β-nicotinamide adenine dinucleotide phosphate (reduced form, NADPH), hydrogen peroxide (30% solution), D-mannitol, adenosine 5′-diphosphate (ADP), succinate, 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron), superoxide dismutase (SOD, type I, from bovine liver), lucigenin, diphenyleneiodonium (DPI), dextran (T500), cytochalasin B, formyl-Met-Leu-Phe (FMLP), A23187 and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (USA). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was obtained from Wako (Japan). Ficoll-Paque was obtained from Pharmacia Biotech. Trolox was purchased from Aldrich (USA). Penicillin, streptomycin, DMEM medium, Hanks' balance salt solution (HBSS), glutamine and fetal calf serum (FCS) were obtained from Gibco. Rhodamine 123 (Rh123) and propidium iodide (PI) were purchased from Molecular Probes (USA). All the other chemicals used in this study were of reagent grade.

2.2. Antioxidant activity in rat brain homogenate, hepatic microsome and mitochondria

Rat brain homogenates and hepatic microsomes were prepared from the brains and livers of freshly killed Wistar rats and their peroxidation induced by the different methods was measured by the thiobarbituric acid (TBA) method, as described by Teng et al. [28] and Hsiao et al. [29]. DMSO, the solvent for cinnamophilin, had been controlled at a constant final concentration (0.5% and 0.25% v/v, respectively). Tetramethoxypropane was used as a standard, and the results were expressed as nanomoles of malondialdehyde equivalents per milligram of protein of both preparations. Furthermore, the activity of microsomal NADPH-cytochrome *P*-450 reductase was evaluated spectrophotometrically by the rate of cytochrome *c* reduction as previously described [29].

Mitochondria were prepared by conventional differential centrifugation [30] with some modification from the livers of Wistar male rats (200–250 g) fasted overnight. The livers were washed and placed in ice-cold isolation buffer containing 70 mM sucrose, 220 mM mannitol, 3 mM EDTA, 0.1 mg/ml BSA and 1 mM Tris-HCl, pH 7.4. Each liver was finely minced with chilled sharp scissors, then homogenized by hand in a glass Dounce homogenizer at a 15% (w/v) concentration. The homogenate

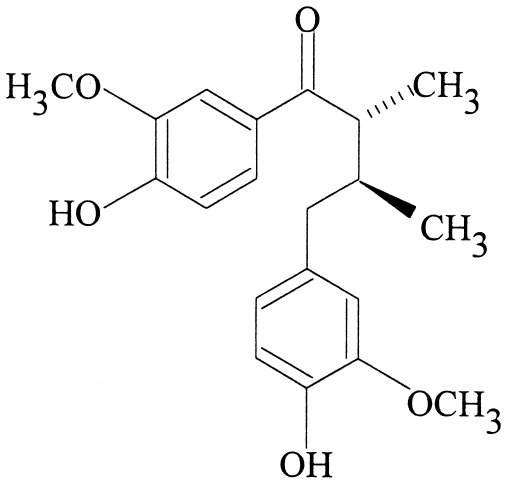

Fig. 1. Chemical structure of cinnamophilin.

was centrifuged at 1900 rpm for 10 min. The supernatant medium was carefully removed by decantation (leaving behind a white fluffy layer) and centrifuged for 7 min at 10 000 rpm to sediment the mitochondria. All centrifugation steps were performed at 4°C in a Sorvall RC5c centrifuge with an SM-24 rotor (DuPont, Wilmington, DE). The supernatant and the upper light-colored layer of sediment were then decanted as cleanly as possible. The red-brown lower layer was gently suspended and centrifuged for 7 min at 10 000 rpm. After final isolation, the mitochondrial pellet was gently suspended in the respiratory buffer (154 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 5 mM potassium phosphate buffer, pH 7.4) to give a 40–50 mg/ml suspension and incubated in an ice-cold bath. The respiratory control ratio with succinate (10 mM) as substrate was 3.8 ± 0.1 ($n = 6$) in the respiratory buffer at 30°C. Freshly prepared mitochondria were used in the peroxidative experiments during 2 h.

The test compound or its vehicle (DMSO, 0.125% v/v) was added to the mitochondrial suspension (0.8 mg protein/ml) in the respiratory buffer and 5 min later, the mixture containing Fe^{3+} (20 μM)/ADP (1 mM) was included. Simultaneously, peroxidation was initiated by addition of a final concentration of 0.25 mM ascorbate as reductant and incubated in a gently shaking water bath at 30°C. At the indicated time, the peroxidative reaction was subsequently stopped by the addition of 50 μM BHT and placed in an ice-cold bath. Quantification of TBARS was performed according to the method previously described.

The protein contents of the brain homogenates, liver microsomes, mitochondria and other preparations were determined by the Bio-Rad method [31], using bovine serum albumin as a standard.

2.3. Stable free radical scavenging action

Stable radical scavenging activity was measured according to the method of Mellors and Tappel [32]. An ethanolic solution of the stable nitrogen-centered free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH, 100 μM) was incubated with the test compounds, and the absorbance monitored spectrophotometrically at 517 nm. The concentration ($IC_{0.20}$) of the antioxidant that induced a change in absorbance of 0.20 during 30 min was taken as the potency of free radical scavenging activity.

2.4. Peroxyl radical scavenging action

The scavenging ability of the test compounds on aqueous peroxyl radicals was determined by the method described by Tsuchiya et al. [33]. The stoichiometric factors of the test compounds with hydrophilic peroxyl radicals were calculated by the equation as mentioned. The rate of peroxyl radical formation from AAPH is 1.6×10^{-6} [AAPH] per second at 40°C [34]. Ascorbic acid or Trolox was used as a positive control.

2.5. Cell cultivation and oxidative treatment

A7r5 cells (rat aortic smooth muscle cell line), obtained from the Culture Collection and Research Center (CCRC, No. 60082, Taiwan), cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 20 mM HEPES, 10% heat-deactivated FCS, 1% (w/v) penicillin/streptomycin and 2 mM glutamine at 37°C in a humidified atmosphere with 5% CO_2. For subculturing, confluent dishes were detached by 0.05% trypsin in 0.53 mM EDTA solution (Gibco), then neutralized with FCS, at a split ratio of 1:4 every 4 days. Trypsinized A7r5 cells were seeded at a density of $8.5 \times 10^3/cm^2$ in Corning 100×20 mm flat-bottomed tissue culture petri dishes. Throughout the experiments, cells were used between passages 11 and 16 from the origin of CCRC given. Before the experiments, the confluent cell suspension was created by resuspension in ice-cold Hanks' balanced salt solution (Ca^{2+}/Mg^{2+}-free HBSS, without divalent cations and phenol red, Gibco) containing 0.8% BSA in pH 7.4 at a density of about 1×10^6 cells/ml. The cell suspension was divided into two parts. For the determination of mitochondrial membrane potential, a part of the A7r5 suspension was then rewarmed and stained with rhodamine 123 (Rh123, 14 μg/ml) and kept in the dark at 37°C for 30 min. The cells were washed once with an adequate volume of cold Ca^{2+}/Mg^{2+}-free HBSS containing 0.8% BSA. After centrifugation, cells were resuspended in the buffered solution with 1 mM Ca^{2+} and Mg^{2+} at the same density as previously described.

For oxidative injury studies, both rhodamine 123- and non-dye-labeled A7r5 cells were treated with vehicle (DMSO, 0.1%) or cinnamophilin (20 and 50 μM) for 10 min before exposure (except control) to the oxidative system consisting of 500 μM of alloxan, 200 μM of ferrous ions and 3 mM of H_2O_2. The reaction mixture was incubated at 37°C in a shaking water bath for the indicated

times (1, 2 and 3 h). For sample analysis, an aliquot of rhodamine-labeled cells was mixed with ice-cold PBS containing 1% BSA (PBS/BSA, 0.7 ml) and passed through a nylon strainer (mesh diameter of 40 μm, Falcon) before introduction into the flow cytometer. On the other hand, PI (50 μg/ml, 75 μl) was added to an aliquot of non-dye-labeled cells (0.3 ml) with rapid mixing to give a final concentration of 10 μg/ml and incubated in the dark at 4°C for 10 min, then an extra 0.7 ml of PBS/BSA was added and the aliquot was passed through the strainer before its application to the flow cytometer.

2.6. Flow cytometric analysis

The fluorescent intensities of Rh123 and PI for samples of 5000 cells were analyzed by flow cytometry with a FACScan flow cytometer (Becton Dickinson). Forward and side scatters were gated on the major population of normal-sized A7r5. The fluorescences of Rh123 and PI were collected with different band pass filters as described [35]. Light scatter values were measured on a linear scale of 1024 channels and fluorescent intensities on a logarithmic scale. Data are expressed as mean channel fluorescence for each sample as calculated by the CellQuest software (Becton Dickinson) on a Power Macintosh 7300/200 computer.

2.7. Human LDL isolation and oxidation

Human LDLs ($d = 1.019$–1.063 g/ml) were isolated from freshly citrated plasma of healthy donors by differential ultracentrifugation [36]. The protective effects of test compounds on copper-catalyzed LDL oxidation were determined by the method previously described [37]. As an assessment of lipid peroxidation, the formation of TBARS was determined and expressed as MDA equivalents per milligram of LDL protein. The extent of aldehyde-modified lysine in oxidized LDL was also monitored by determining the fluorescence intensity (excitation at 350 nm, emission at 420 nm).

2.8. Quenching of superoxide anions

The superoxide scavenging activity of the compounds was determined by spectrophotometrically monitoring their competition with cytochrome *c* for superoxide anion generated by the xanthine/xanthine oxidase method [28]. The initial rate of superoxide-induced ferricytochrome *c* reduction was determined by subtracting the cytochrome *c* reduction in the presence of 100 units/ml superoxide dismutase. The results were expressed as the percent inhibition of initial rate on cytochrome *c* reduction.

The quenching of superoxide was also precisely evaluated by the chemiluminescence method. Various test compounds were preincubated with xanthine (50 μM) and lucigenin (60 μM) in 50 mM KH_2PO_4-KOH solution at 37°C for 3 min, then elicited by xanthine oxidase (0.0167 units/ml). Chemiluminescence was continuously measured in a thermostatic PC-controlled luminometer analyzer (Bio-Orbit 1251, Turku, Finland), using disposable polystyrene cuvettes. The integrated chemiluminescence was determined in the first period of 30 s before (B_{30}) and after (A_{30}) xanthine oxidase added. The results were expressed as the ratio of the integrated luminescence (A_{30}/B_{30}). Reference compounds such as superoxide dismutase and non-enzymatic superoxide scavenger Tiron [38] were also investigated.

Under the same condition, the effects of test compounds on the enzymatic activity of xanthine oxidase were determined by spectrophotometrically measuring uric acid formation as previously mentioned [28].

2.9. Vascular superoxide anion quenching action

The superoxide anion production of the aortic rings was measured by the method of lucigenin-enhanced chemiluminescence as described by Rajagopalan et al. [39] with some modifications. The experiments were performed using aortae obtained from 20–24 week old SHR and Wistar Kyoto rats (WKY). Systolic blood pressures of SHR were measured by tail cuff plethysmography (Narco Biosystems) as 174.5 ± 5.5 mmHg before surgical procedures. The rat thoracic aorta was carefully removed and placed in chilled, modified Krebs/HEPES buffer consisting of 118.0 mM NaCl, 20.0 mM HEPES, 4.7 mM KCl, 1.0 mM $MgSO_4$, 25.0 mM $NaHCO_3$, 1.0 mM KH_2PO_4, 1.8 mM $CaCl_2$ and 5.5 mM glucose, pH 7.4, then cleaned of excessive adventitial tissue, and cut into 5 mm ring segments. The aortic rings were then transferred to an organ bath containing modified Krebs/HEPES buffer, maintained at 37°C and gassed with 95% O_2/5% CO_2 to equilibrate for 30 min. Therefore, rings with vehicle (DMSO) or test compounds were placed in the wells of a standard scintillation microplate (EG&G Berthold, Wildbad, Germany) containing 200 μl modified Krebs/HEPES buffer and subsequently transferred to a PC-controlled LB96V chemiluminescence analyzer with WinGlow software (EG&G Berthold) thermostatically controlled at 37°C for 3 min. Then, 50 μl aliquots of lucigenin were added at a final concentration of 250 μM by the jet-injection system. Scintillation counts were recorded by the new state-of-the-art ultrafast single photon counter and integrated over a 0.1 s interval for 15 min, and the average integrated background counts (wells without aortic rings) were subtracted. All vessels were then dried by placing them in a 90°C oven for 24 h, for determination of dry weight. Calibration of lucigenin chemiluminescence was constructed using the rates of superoxide generation from 1–10 mU/ml xanthine oxidase plus 100 μM xanthine, as determined by ferricytochrome *c* reduction as previously described. Tiron, a cell-permeant scavenger of superoxide anion, and diphenyleneiodonium (DPI), an inhibitor

of NADPH oxidase [40], were used as the positive controls.

2.10. Preparation of human neutrophils and measurement of superoxide anion production

Citrated blood samples were obtained from healthy individuals by venipuncture after informed consent had been given. Neutrophils were isolated by sedimentation through dextran (6% w/v), centrifugation through Ficoll/Hypaque gradient medium (Pharmacia), and 20 s hypotonic lysis of erythrocytes. Washed neutrophils were finally resuspended in a modified Hanks' balanced salt solution consisting of 145 mM NaCl, 10 mM K_2HPO_4, 10 mM HEPES, 4.2 mM $NaHCO_3$, 5.5 mM glucose and 200 μg/ml human serum albumin, pH 7.4 (mHBSS), and the concentration of neutrophils was measured by a cell counter (Hemalaser, Sebia) or hemacytometer (Hausser) and adjusted to 2×10^7 neutrophils/ml with the same medium. Final preparations contained 97% neutrophil and viability was above 98% as assessed by Riu stain [41] and trypan blue (0.4%, w/v) exclusion, respectively.

Superoxide anion production generated by human neutrophils was determined by measurement of the reduction of cytochrome *c* in the presence or absence of superoxide dismutase as previously described [42]. Briefly, human neutrophils (2×10^6/ml) in mHBSS containing Ca^{2+} (1 mM)/Mg^{2+} (0.5 mM) and cytochrome *c* (40 μM) were combined in a thermostat-controlled stirred cuvette (37°C). After cells were incubated with test compounds or vehicle for 2 min, the reaction was started by the addition of FMLP (100 nM) or PMA (160 nM). In the other conditions, neutrophils were first primed with cytochalasin B (1 μg/ml) for 4 min and treated with test compounds, then triggered by FMLP. The change in absorbance at 550 nm was continuously recorded for 10 min. Results are calculated and expressed as the initial rate of superoxide anion production (nmol/min/10^6 neutrophils) as previously described.

2.11. Statistical analysis

The data are presented as the mean ± S.E. from the number of experiments indicated. Statistical analysis was performed using Student's unpaired *t*-test unless specifically mentioned, and statistical significance was set at $P<0.05$. The IC_{50} value was determined by regression analysis.

3. Results

3.1. Effects of cinnamophilin on lipid peroxidation

Cinnamophilin exerted a concentration-dependent inhibition of iron-catalyzed lipid peroxidation in rat brain homogenates (Fig. 2). At the highest concentration tested (20 μM), cinnamophilin also inhibited spontaneous lipid peroxidation by even more than 90% (data not shown). The antioxidant capacities of cinnamophilin and other classic antioxidants are compared and expressed as IC_{50} in Table 1. Trolox, α-tocopherol and desferrioxamine all suppressed ferrous ion-dependent lipid peroxidation in brain homogenates in a dose-dependent manner. The IC_{50} of cinnamophilin was 8.0 ± 0.7 μM ($n=4$). The potency of cinnamophilin was approximately similar between Trolox and α-tocopherol, and 12-fold that of desferrioxamine. Cinnamophilin (20 μM) itself did not interfere with the absorption at 532 nm when added to rat brain homogenates that were either intact or already oxidatively modified (data not shown).

Table 1
Antiperoxidative effects of cinnamophilin and various antioxidants on ferrous ion-induced lipid peroxidation in rat brain homogenates

Compound	IC_{50} (μM)
Cinnamophilin	8.0 ± 0.7
Trolox	13.4 ± 0.6
α-Tocopherol	3.8 ± 0.4
Desferrioxamine	95.9 ± 4.7

The antioxidant activity of test compounds was calculated as percent inhibition of ferrous ion (200 μM)-induced lipid peroxidation. Concentrations causing 50% inhibition (IC_{50} values) are presented as means ± S.E. from four independent experiments.

When the peroxidative insult as 20 μM Fe^{2+}/1 mM ADP and 0.25 mM ascorbate was simultaneously added to the rat liver mitochondria, a time-dependently intense lipid peroxidation resulted as measured by the formation of TBARS for 15 min, 30 min and 60 min (17.6 ± 0.7, 26.1 ± 0.2 and 27.6 ± 0.8 nmol MDA equivalent/mg protein, respectively, $n=3$). As shown in Fig. 2, cinnamophilin inhibited the formation of TBARS on mitochondria in a concentration-dependent manner with an IC_{50} value of 17.7 ± 0.2 μM ($n=3$). The progression of mitochondrial peroxidation was completely suppressed even up to 95% after 1 h by the treatment with cinnamophilin at a concentration of 50 μM.

Enzyme-catalyzed lipid peroxidation of rat liver microsomes was triggered in the presence of NADPH as the reductant in the microsomal electron transfer system and ferric ADP as the iron catalyst. As shown in Fig. 2, cinnamophilin suppressed this peroxidative reaction in a concentration-dependent manner with an IC_{50} value of 3.4 ± 0.1 μM ($n=4$). Cinnamophilin at a concentration of 10 μM almost abolished this enzyme-catalyzed peroxidation. Additionally, the classic antioxidants such as α-tocopherol, Trolox and desferrioxamine at the same concentration exerted inhibitions of 59.3 ± 2.6, 40.2 ± 1.2 and 0.8 ± 0.6%, respectively ($n=3$). To evaluate the effect of cinnamophilin on the activity of microsomal NADPH-cytochrome *P*-450 reductase we used cytochrome *c* as a substrate. Both the rates of cytochrome *c* reduction in the vehicle-treated and cinnamophilin-treated (10 μM) groups

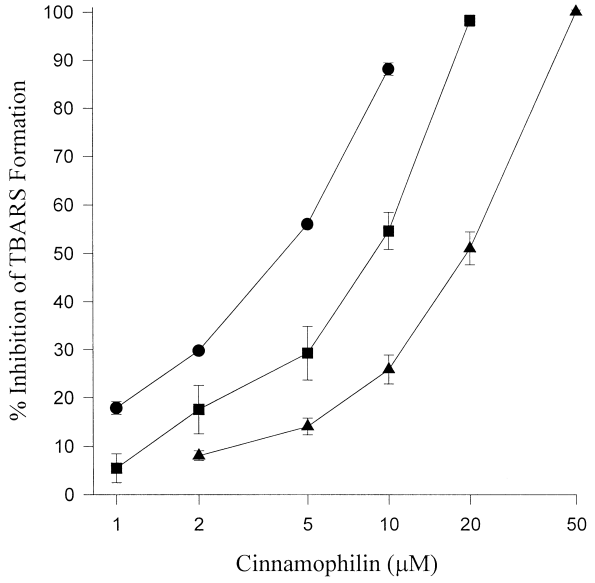

Fig. 2. The inhibitory effects of cinnamophilin on enzymatic and non-enzymatic lipid peroxidation. Rat liver microsome (●), brain homogenate (■) and mitochondria (▲) were preincubated with DMSO (0.25, 0.5 and 0.125% v/v, respectively) or various concentrations of cinnamophilin at 37°C for 10 min, then 0.4 mM NADPH/100 μM, Fe^{3+}/4 mM ADP, 0.2 mM Fe^{2+} or 0.25 mM ascorbate/20 μM Fe^{3+}/1 mM ADP was added and incubation continued for another 30 min, respectively. In controls, TBARS formation was 10.0 ± 0.4, 10.5 ± 1.2 and 26.1 ± 0.2 nmol MDA/mg protein in rat liver microsomes, brain homogenates and mitochondria, respectively. Values are expressed as the percent inhibition of TBARS formation. Each point represents the mean ± S.E. from three to four experiments.

were not significantly different (87.0 ± 1.8 and 86.7 ± 0.6 nmol cytochrome *c*/min/mg protein, respectively; $n = 3$–4). According to these results, the inhibitory effect of cinnamophilin on microsomal lipid peroxidation was not due to impairment in the electron chain flow of NADPH-cytochrome *P*-450 reductase.

3.2. Stable free radical (DDPH) scavenging action

Diphenyl-*p*-picrylhydrazyl decolorization was used to evaluate the ability of compounds to act as free radical scavengers. The scavenging activity of cinnamophilin expressed as the $IC_{0.2}$ value was approx. 6.2 ± 0.8 μM ($n = 6$). It was nearly more potent than probucol, α-tocopherol, Trolox, ascorbate and BHT, with $IC_{0.2}$ values of 9.6 ± 0.5, 7.1 ± 0.2, 9.4 ± 0.3, 11.1 ± 1.1 and 12.1 ± 0.8 μM ($n = 4$–6), respectively. Interestingly, the biphasic pattern of decolorization induced by cinnamophilin was similar to that by Trolox or α-tocopherol, but not by butylated hydroxytoluene (data not shown).

3.3. Peroxyl radical scavenging action

Exposure of B-phycoerythrin to AAPH-derived aqueous peroxyl radicals induced a transient decay of the fluorescent intensity. Cinnamophilin produced a concentration-dependent (1, 2 and 5 μM) decrement of the fluorescence loss and prolongation of the lag time (Fig. 3). The stoichiometric factor of cinnamophilin for scavenging peroxyl radicals was calculated to be 2.1 ± 0.1 ($n = 5$), while those of ascorbate and Trolox were 0.9 ± 0.1 ($n = 5$) and 2.3 ± 0.1 ($n = 5$), respectively.

3.4. Effect of cinnamophilin on the integrity of cytoplasmic membrane and mitochondria

As observed by the flow cytometric method, the occurrence of changes in forward angle light scatter is shown by the alteration of cellular deformation. When alloxan/Fe^{2+}/H_2O_2 was used to challenge A7r5 (rat aortic smooth muscle cell line), they showed an elevation of the mean forward scatter value within 1 h. However, such an increment of the forward scatter value was dose-dependently inhibited by cinnamophilin (20 and 50 μM) (data not shown). In the fluorescent studies, incubation of A7r5 with alloxan/Fe^{2+}/H_2O_2 resulted in a time-dependent increment in membrane permeability and disruption in the mitochondrial transmembrane potential as evidenced by a change in cellular PI and Rh123 fluorescence, respectively (Fig. 4). The intensity of PI fluorescence was time-dependently increased following addition of the prooxidant insult. In this setup, the start of oxidative damage on cytoplasmic membrane was prior to 1 h. Also, according to the fluorescence histogram, prolonged time exposure produced a time-dependent increment in the number of cells stained with PI. The fraction of the PI-labeled cells of total cells during exposure to prooxidants for 1, 2 and 3 h was 47.9 ± 5.4, 72.4 ± 1.5 and 82.7 ± 1.6%, respectively ($n = 4$). However, the cell suspensions used throughout the experi-

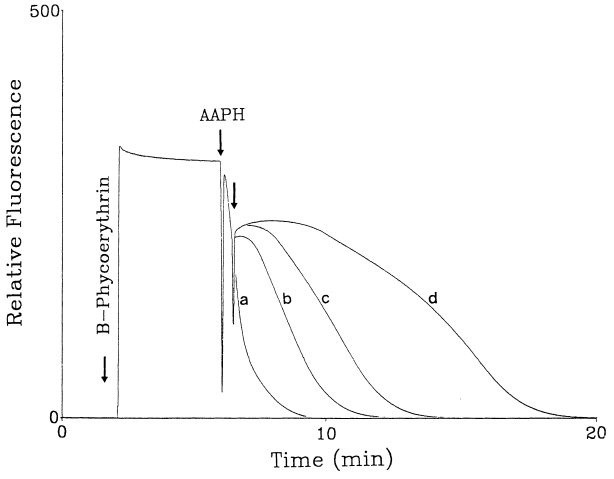

Fig. 3. Effect of cinnamophilin as a protectant against the hydrophilic peroxyl radical-induced degradation of B-phycoerythrin. The fluorescence intensity of B-phycoerythrin was measured with 540 nm excitation and 575 nm emission. The moment of the addition of cinnamophilin (1 μM, b; 2 μM, c; 5 μM, d) or DMSO (0.5%, a) to the reaction mixture is indicated by the arrow. Representative tracings of four independent experiments are shown.

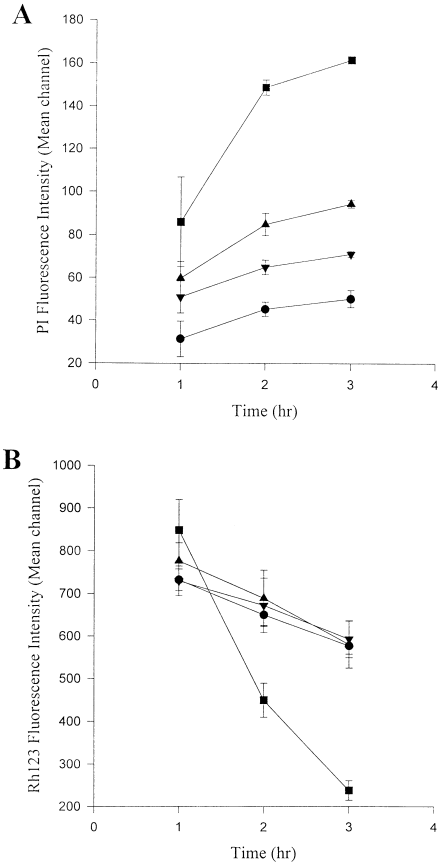

Fig. 4. The effect of cinnamophilin on oxidative insult in A7r5 cells. Kinetics of (A) cell membrane disturbance and (B) mitochondrial transmembrane potential collapse was determined by the fluorescent intensity of PI or Rh123, respectively, by a benchtop flow cytometer. Both measurements were performed as described in Section 2. The oxidative insult of A7r5 was carried out by addition of alloxan/Fe^{2+}/H_2O_2 in the absence (■) or presence of 20 μM (▲) or 50 μM (▼) cinnamophilin in humidified air at 37°C in a shaking bath. ●, resting condition, treated without any oxidative challenge. Data are shown as means ± S.E. from three to four independent experiments.

ments contained PI-stained cells which were less than 21% of total cells. It was clearly shown that the elevation of cellular PI fluorescence intensity as cellular membrane disruption was markedly suppressed by cinnamophilin in a time- and concentration-dependent manner (Fig. 4A). The increase of PI fluorescence of A7r5 was inhibited up to approx. 80% even after 3 h by the treatment with cinnamophilin at a concentration of 50 μM. On the other hand, as observed in the fluorescence histogram, this lignan also attenuated an increase in the fraction of total cells stained with PI during exposure to prooxidants (data not shown). Rh123, as a cationic lipophilic dye, has been used as a sensitive fluorescent probe to determine the transmembrane mitochondrial potential. The oxidative stress-induced kinetic pattern of mitochondrial Rh123 fluorescence decay is consistent with the observation of another laboratory [43] that the alterations in mitochondrial potential are reflected as an initial increase in fluorescence as the H-aggregates are lost, followed by a decrease as the dye is lost from the organelle. As shown in Fig. 4B, the Rh123 fluorescent intensity of A7r5 was elevated within the first hour, then a time-dependent decay followed upon the addition of alloxan/Fe^{2+}/H_2O_2. Cinnamophilin produced a concentration-dependent inhibition of the elevation of Rh123 fluorescent intensity within the first hour. The following sharp loss of Rh123 fluorescent intensity triggered by an oxidative impulse was also completely restored by cinnamophilin even at a concentration of 20 μM. According to the Rh123 fluorescence histogram, prolonged exposure to prooxidants produced a time-dependent decrease in the fraction of Rh123-stained cells of total cells. After 3 h oxidative insult, the fraction of cells that maintained an intact Rh123-stained condition decreased to 27.3 ± 5.3% of total cells, as compared to the control: 82.5 ± 1.3% of total cells. Under the same condition, cinnamophilin produced a dose-dependent (20 and 50 μM) increase in the fraction of intact Rh123-stained cells as 63.8 ± 3.5 and 72.3 ± 4.5% of total cells, respectively.

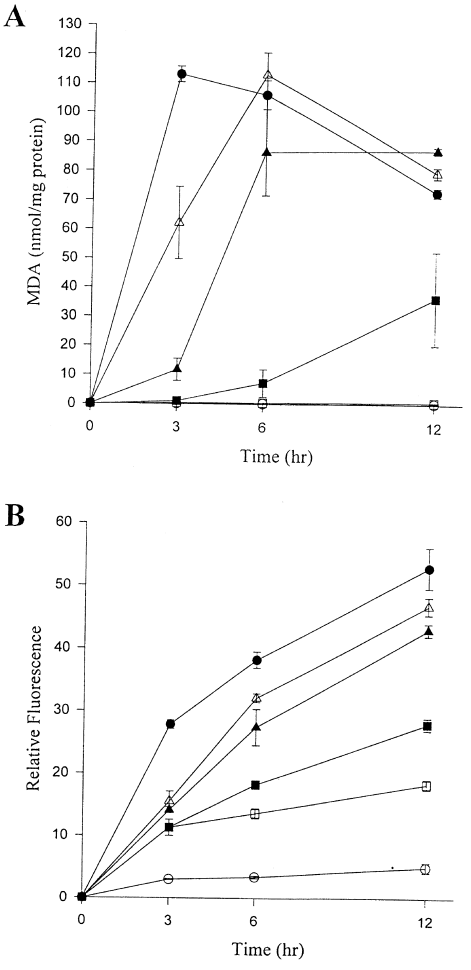

Fig. 5. Effects of cinnamophilin on (A) thiobarbituric acid-reactive substances and (B) fluorescent chromogen formation during human LDL oxidation. The oxidation of LDL (0.1 mg/ml) was carried out by addition of 5 μM $CuSO_4$ in the absence (●) or presence of 5 (△), 10 (▲), 20 (■) or 50 μM (□) cinnamophilin at 37°C. ○, resting condition. Data are shown as means ± S.E. from four independent experiments.

3.5. Inhibition of copper-catalyzed LDL oxidation

The treatment of human LDL with Cu^{2+} at a concentration of 5 μM had a time-dependent influence on the formation of oxidative modification of LDL, as evidenced by the elevation in lipid peroxidative products (i.e., TBARS) and the modification of apolipoprotein B (i.e., fluorescent chromogen) shown in Fig. 5A and B, respectively. Cinnamophilin exerted an inhibitory action on this transition metal ion-catalyzed lipid peroxidation: the formation of TBARS and the elevated intensity of fluorescence were reduced in a concentration-related manner (Fig. 5). Furthermore, both peroxidative parameters of LDL oxidation were significantly suppressed by cinnamophilin at a concentration of 50 μM even for 12 h. It was remarkable that the inhibitory effects of cinnamophilin on these markers (i.e., TBARS and fluorescent chromogen) were well correlated simultaneously, and that this lignan-derived substance itself also did not quench the fluorescence when added to an LDL preparation which was already oxidatively modified.

3.6. Superoxide anion quenching action

By the spectrophotometrical method, the initial rate of superoxide-induced cytochrome *c* reduction was diminished either by cinnamophilin or Tiron in a concentration-dependent manner (Fig. 6A), and the production of superoxide anions was completely inhibited by cinnamophilin at 200 μM. However, Tiron induced a 62.0 ± 1.0 inhibition at the same concentration ($n = 4$). Thus, it was clearly shown that cinnamophilin was more potent than Tiron on the inhibition of superoxide production. In the presence of SOD (10 units/ml) or allopurinol (100 μM), cytochrome *c* reduction was abolished by 86.7 ± 0.7 or 63.8 ± 1.0%, respectively ($n = 4$). In addition, under the similar superoxide generation system, the sensitive short-time lucigenin-enhanced chemiluminescence was also inhibited by cinnamophilin in a dose-dependent manner and its inhibitory biphasic trace pattern was similar to that of the combination of Tiron and allopurinol (data not shown). Therefore, cinnamophilin concentration-dependently (20, 50, 100 and 200 μM) decreased the value of the ratio of integrated chemiluminescence from the stimulated value of 87.4 ± 1.1 to 38.5 ± 0.6, 25.1 ± 0.8, 13.0 ± 0.6 or 6.2 ± 0.2, respectively (Fig. 6B). The reference compounds SOD (10 units/ml), Tiron (100 μM) and allopurinol (100 μM) exerted ratio values of 1.7 ± 0.1, 4.4 ± 0.1 and 63.9 ± 1.1 ($n = 4$–5).

The enzymatic activity of xanthine oxidase was attenuated by cinnamophilin in a concentration-dependent manner (5.3 ± 0.3, 16.4 ± 0.2, 24.0 ± 0.9 and 32.0 ± 1.9% inhibition for 20, 50, 100 and 200 μM cinnamophilin, respectively; $n = 3$–5). Allopurinol (100 μM), as a positive control, elicited an inhibition of 81.3 ± 1.3% ($n = 7$). These results revealed that cinnamophilin is a superoxide anion scavenger with a minor action on xanthine oxidase.

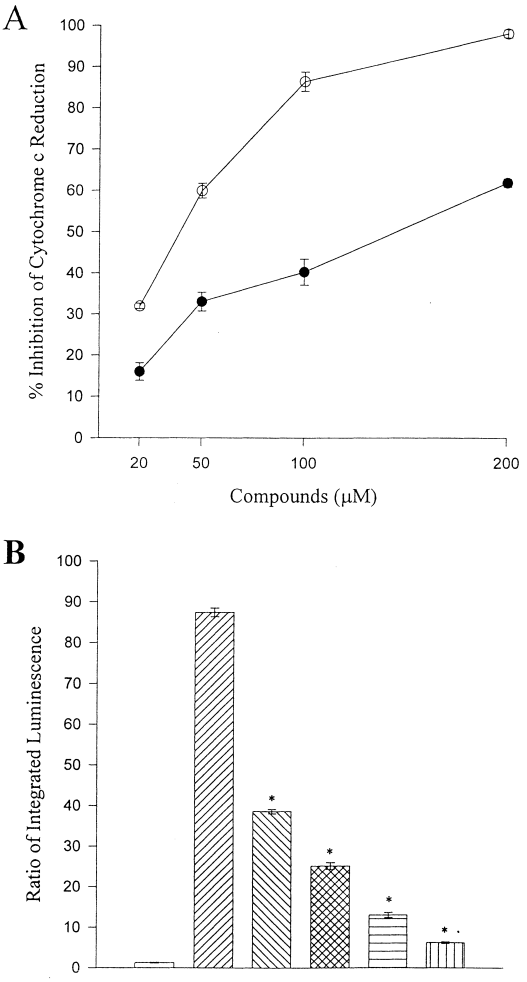

Fig. 6. Effects of cinnamophilin on cell-free xanthine/xanthine oxidase-induced cytochrome *c* reduction and lucigenin-enhanced chemiluminescence. (A) Various concentrations of cinnamophilin (○), Tiron (●) or DMSO (0.5%, control) were preincubated with xanthine (100 μM) at 25°C for 3 min in the presence of cytochrome *c* (0.5 mg/ml) in 50 mM KH_2PO_4/K_2HPO_4 solution (pH 7.4), and catalyzed with 0.02 U/ml xanthine oxidase. Cytochrome *c* reduction was monitored continuously as described in Section 2. Values are expressed as percent inhibition of initial rate of cytochrome *c* reduction. Each point represents the mean ± S.E. from four to seven independent experiments. (B) Cinnamophilin (hatched from left to right, 20 μM; cross-hatched, 50 μM; horizontally striped, 100 μM; vertically striped, 200 μM) or DMSO (hatched from right to left, 0.5%) were preincubated with xanthine (50 μM) for 3 min in 50 mM KH_2PO_4-KOH solution in the presence of 60 μM lucigenin, then stimulated with xanthine oxidase (0.0167 U/ml). Empty bar, resting condition, mixture treated without any stimulation. Lucigenin-enhanced chemiluminescence was continuously detected and analyzed by the integration mode as described in Section 2. Data are given as means ± S.E. from six to ten independent experiments. *$P < 0.001$ when compared with the control.

3.7. Effects of in vitro cinnamophilin treatment on superoxide production in aortas and neutrophils

Superoxide anions generated by aortic rings were

assessed by lucigenin-enhanced chemiluminescence. Superoxide production was greater in aortae from aged SHR than in those from WKY (160 ± 23.4 vs. 106.1 ± 22.1 pmol/15 min/mg tissue, respectively, $n = 4$). As shown in Fig. 7A, treatment of aortic segments of SHR with cinnamophilin (100 μM) had a significantly inhibitory effect on the production of superoxide anion, which was practically abolished in the presence of the cell-permeant superoxide scavenger Tiron (5 mM) or the NADPH oxidase inhibitor diphenyleneiodium (50 μM). Additionally, the inhibitory effect of cinnamophilin on superoxide production of human neutrophils elicited by FMLP and PMA is shown in Fig. 7B. The initial rate of human neutrophil superoxide generation induced by 100 nM FMLP was 2.33 ± 0.11 nmol/min/10^6 cells, and that triggered by 160 nM PMA was 10.27 ± 0.82 nmol/min/10^6 cells ($n = 3$). Cinnamophilin (20 μM) could completely inhibit FMLP-induced superoxide production. Interestingly, it also reduced the enhanced production of superoxide from cytochalasin B (1 μg/ml)-primed neutrophils even up to 90% inhibition (data not shown). And, cinnamophilin could partially suppress the calcium ionophore (A23187)-induced respiratory burst even up to a 35% inhibition at a concentration of 20 μM (data not shown). However, the kinetic pattern and initial rate of superoxide formation induced by PMA were slightly but not significantly affected by cinnamophilin at the same concentration. The superoxide production induced by these two agonists was completely abrogated by either superoxide dismutase (200 U/ml), DPI (20 μM) or Tiron (1 mM) (data not shown).

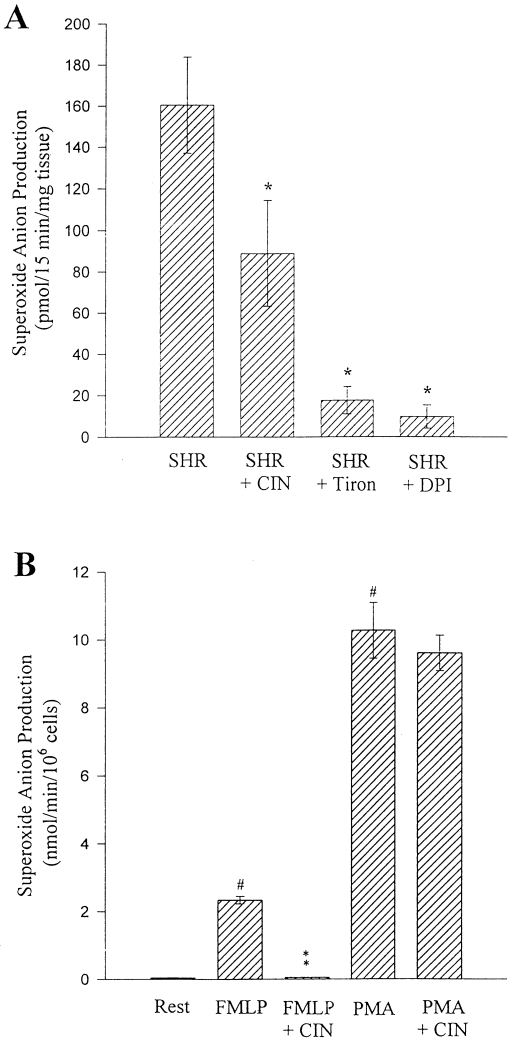

Fig. 7. Effects of cinnamophilin on superoxide anion production in rat aortas and human neutrophils. (A) Vascular superoxide anion production assessed by lucigenin-enhanced chemiluminescence. Aortic segments from aged SHR were treated with vehicle (DMSO, 0.2%), cinnamophilin (CIN, 100 μM), Tiron (5 mM) and diphenyleneiodium (DPI, 50 μM), respectively. Lucigenin-enhanced chemiluminescence was measured, and units of chemiluminescence were converted to nmoles of superoxide by standardizations as described in Section 2. Results are expressed as the means ± S.E. from four separate experiments. Statistical analysis was performed by paired Student's t-test. $^*P < 0.05$. (B) Neutrophils were preincubated with vehicle (DMSO, 0.05%) or cinnamophilin (CIN, 20 μM) for 3 min, and stimulated with fMet-Leu-Phe (FMLP, 100 nM) or 12-myristate 13-acetate (PMA, 160 nM). The initial rate of superoxide anion generation was determined by SOD-sensitive cytochrome c reduction assay. Results are given as means ± S.E. from three independent experiments. $^{\#}P < 0.001$ when compared with the resting condition. $^{**}P < 0.001$ when compared with the respective control.

4. Discussion

Both non-enzymatic and enzymatic lipid peroxidation generally occur by the oxidative insult on the phospholipid bilayers of cellular and subcellular membranes in some pathological conditions [5,44]. Therefore, a compound with antiperoxidative activity seems to exert a pharmacological benefit in the protective and therapeutic implications of radical-induced pathological events [45]. The induction mechanism of non-enzymatic lipid peroxidation was proposed that ferrous iron or the ascorbate-mediated ferrous ion-ADP complex site-specifically bound into the vicinity of membrane phospholipids and directly led to a peroxidative chain reaction [5]. Additionally, enzymatic microsomal NADPH-dependent lipid peroxidation is triggered by electrons from NADPH passing directly themselves or indirectly by superoxide to some ferric ions, then generating ferrous ions and eventually elicit lipid peroxidation [46]. In the studies of non-enzymatic lipid peroxidation, we found that the natural lignin compound cinnamophilin could concentration-dependently inhibit iron-induced lipid peroxidation. And it was more potent than desferrioxamine and equipotent with other classic antioxidants such as Trolox and α-tocopherol in the brain homogenate assay. Furthermore, cinnamophilin exerted the most potent inhibitory effect on microsomal enzymatic lipid peroxidation. This also implies that cinnamophilin had no ascorbate-like prooxidant capacity in the presence of iron [47] and could reach the vicinity of membrane phospholipid where the site-bound iron ion is located during lipid peroxidation. According to the potent iron chelator with ferroxidase activity, desferrioxamine [48], when

inhibited 50% lipid peroxidation, its concentration was approximately the half molar equivalent of the total added iron in the brain homogenate study. However, cinnamophilin exerted an inhibitory degree of 50% at a concentration 25-fold less than that of the exogenously iron added in the assay. Hence, it is conceivable that the iron ion chelating property of cinnamophilin did not predominantly contribute to the antiperoxidative activity under this experimental method. Therefore, cinnamophilin acts as an efficient membranous antioxidant in brain homogenate (mixed membranous system), microsome (uniform organelle membranous system) and mitochondria (intact organelle).

The diphenyl-*p*-picrylhydrazyl tests provided direct information about the fact that cinnamophilin acted as a free radical scavenger and that its potency was equipotent to α-tocopherol but more potent than other classic antioxidants. Furthermore, it is well known that the generation of peroxyl radicals is a necessary proximate step in the formation of TBARS during lipid peroxidation [44]. According to the stoichiometric factor calculated, cinnamophilin was able to interact with peroxyl radicals with scavenging efficacy at a mole-to-mole stoichiometric ratio of 2 and was more potent than ascorbate and equipotent to Trolox. It is reasonable to assume that the two hydrogen donating HO-groups of cinnamophilin may contribute to its peroxyl radical trapping activity. Since transition metals such as iron were not used in this peroxyl radical generative system, the scavenging activity of cinnamophilin was not due to the property of transition metal chelation. From these aforementioned findings, cinnamophilin is a strong and versatile antioxidant, and its antiperoxidative mechanism is mainly through its free radical scavenging activity. However, whether cinnamophilin possesses a membrane stabilizing or recycling activity needs further investigation.

Recently, pathological findings showed that loss of smooth muscle cells is associated with atherosclerotic plaque instability [49] and diabetic retinal microvascular lesions [50]. Under some circumstances, overwhelming oxidants and impaired antioxidant status could be found in the vascular cells during cytotoxic cascade [51,52]. The mechanism of H_2O_2-mediated cytotoxicity is supposedly through the formation of a highly reactive hydroxyl radical in the presence of transition metal ions [53]. H_2O_2 could not only cause lipid peroxidation of the cellular membrane bilayer, but also induce intracellular Ca^{2+} overload and poly(ADP-ribose) synthetase activation, which are responsible for mechanical and metabolic damage [54,55]. Especially, alloxan as a diabetogenic agent also exerts actions on the redox reaction for superoxide anion production and is implicated in DNA damage [56]. From our experimental results, we found that A7r5 cells insulted with alloxan, ferrous ion and hydrogen peroxide exerted the enhancement of cell death as compared to each single insult treated. Thus, this aggressive attack on A7r5 cells was clearly demonstrated by the cellular intense alteration of mitochondrial potential and cytoplasmic membrane disruption. And as the results show, it is conceivable that the cascade of multiple oxidant-induced A7r5 cytotoxicity was first to disturb cytoplasmic membrane and thereafter alter mitochondrial integrity. Cinnamophilin exerted significant protection against cytotoxic damage on the biomembrane and mitochondria of A7r5 cells. And it is different from some antioxidants such as gallic acid and butylated hydroxytoluene which could cause lethal cell injury [57,58]. On the other hand, prior cell-free studies imply that the antioxidant properties of cinnamophilin may not merely depend on interaction with cytosolic redox constituents in the cells. These data suggest that cinnamophilin exerts antioxidant activity in the cells and hence raises the availability against external oxidant insult under pathophysiological conditions.

The oxidative modification hypothesis is strongly supported by the evidence that LDL oxidation occurs in vivo and contributes to the clinical manifestations of atherosclerosis [12]. Cinnamophilin caused, in a concentration-dependent manner, a prolongation of the peroxidative lag time in human LDL exposed to copper ions. From the previous results, this natural compound acts as a chain breaking antioxidant in the different membranous systems and intact cells. Therefore, it implies that cinnamophilin could also reach the site of LDL particles where copper is associated, thus protecting LDL against the oxidative modification of LDL. On the other hand, the possibility of the anti-LDL oxidation action through regeneration of endogenous antioxidants as similar to the recycling mechanism for α-tocopherol by ascorbate [59] needs further investigation.

Reactive oxygen species such as superoxide anions play an important role in the pathogenesis of cardiovascular disorders [18]. It is well known that NADPH/NADH oxidase and xanthine oxidase are the two major sources of superoxide anions in the cardiovascular system [39,60]. Especially, the generation of superoxide anions during early reperfusion of ischemic tissues is mediated mainly through the activation of xanthine oxidase [61]. Cinnamophilin could concentration-dependently scavenge xanthine/xanthine oxidase-generated superoxides under two different assay systems, but it also had a weakly inhibiting action on xanthine oxidase. Furthermore, cinnamophilin was more potent than the non-enzymatic superoxide scavenger Tiron. Therefore, cinnamophilin is possibly beneficial in the micromolar range for preventing superoxide-induced damage in pathological conditions. Substantial evidence also indicates that the major source of abnormal vascular superoxide anions from NADPH oxidase is a characteristic of several vascular diseases [62,63]. We found that cinnamophilin, DPI and Tiron were effective in inhibiting the elevated vascular lucigenin chemiluminescence signal. It is consistent with other reports [17] that aortic vessels from aged SHR could release an abundance of superoxide

anions by NADPH oxidase. However, the vascular superoxide scavenging activity of cinnamophilin was less effective than the previous superoxide assessment system. It is conceivable that cinnamophilin, due to its hydrophobic nature, could be preferentially localized in close proximity to the vascular connective tissues. On the other hand, according to neutrophil activation studies, we found that cinnamophilin inhibited respiratory burst possibly not through either inhibition of protein kinase C or merely calcium mobilization but through an upstream signal pathway. It seems reasonable to assume that cinnamophilin diminished superoxide production from aortic segments or activated neutrophils maybe not through inhibition on NADPH oxidase. These results also imply that cinnamophilin works bifacially as an antioxidant and anti-inflammatory agent through either scavenging or anti-neutrophil actions.

In conclusion, this study shows that cinnamophilin is a novel natural antioxidant against lipid peroxidation in various membranous systems. The antioxidant activity of cinnamophilin acts predominantly via versatile free radical scavenging action. Furthermore, it is a novel cytoprotectant against oxidative stress and effective in inhibiting oxidative modification of human LDL. Attractively, cinnamophilin also reduced superoxide anion production from the vessels of aged SHR and respiratory burst of activated human neutrophils. It will be interesting to study further the antioxidant activities of this natural compound in various radical-mediated and inflammatory injuries in pathological events in vivo.

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